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Phenotypic differences in murine chondrocyte cell lines derived from mature articular cartilage

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Summary

Objective: To obtain well characterized immortalized murine chondrocyte cell lines. The cell lines were obtained from mature articular chondrocytes, instead of embryonal cells which are used in most other studies.

Methods: Pieces of articular cartilage were cut from murine patellae and femoral heads. Chondrocytes were isolated by digestion with collagenase. These cells were cultured in monolayer and immortalized by transfection of the SV40 large T antigen gene. To preserve the differentiated phenotype, the resulting clones were cultured in three-dimensional carriers, alginate beads. The phenotypes of the cells were characterized using the following parameters: Cell morphology (light microscopy), messenger RNA (RT-PCR) and protein (immunohistochemistry) levels of extracellular matrix molecules. Moreover, responsiveness to interleukin-1(IL-1) was determined by measuring production of proteoglycans (^{35}S-sulfate incorporation) and of nitric oxide (Griess reaction).

Results: Sixteen clones were obtained, ten (P1 to P10) derived from patellar cartilage, and six (H1 to H6) from femoral head cartilage. In seven cell lines (P2, P5, H1, H3, H4, H5, H6) high production of type II collagen corresponded with high levels of mRNA of type II collagen (and prevalence of the IIB type) and with high IL-1-induced suppression of proteoglycan synthesis. Like intact murine articular cartilage, all cell lines produced type I and type X collagens, but mRNA levels of both types of collagen were never higher in the cell lines as compared with intact cartilage.

Conclusion: Our results demonstrate that it is possible to immortalize mature murine articular chondrocytes. Each of the obtained chondrocyte cell lines appeared to have a stable phenotype. Both relatively differentiated and relatively dedifferentiated chondrocyte cell lines could be identified.

Key words: Immortalized chondrocyte, Type II collagen, IL-1, Alginate.

Introduction

Investigation of articular chondrocyte responses to cytokines and other environmental signals in health and disease is greatly hindered by the low numbers of chondrocytes available. Moreover, *in vitro* multiplication leads to phenotypic drift, because isolated chondrocytes cultured in monolayer rapidly become fibroblastic, and lose their specific morphological and biochemical characteristics[1–3]. Various directions have been explored to supply large numbers of differentiated articular chondrocytes. One way is immortalization of chondrocytes, but stable retention of the differentiated phenotype has appeared to be difficult to attain[4–7]. A reason for this problem could be the fact that in most immortalization studies embryonal cells were used. Therefore, we decided to use articular chondrocytes from mature mice for immortalization studies. Moreover, cells were encapsulated in alginate beads immediately after the immortalization and cloning procedures. Alginate is a linear polysaccharide (D-mannuronic acid and L-guluronic acid)$_n$, which forms a gel in the presence of calcium or other divalent cations[8]. Freshly isolated articular chondrocytes cultured in alginate beads for 2 weeks synthesize a matrix similar to that of native human cartilage[9,10] and maintain their phenotype for long periods of time[11], as well as their capacity to produce nitric oxide in response to interleukin-1 (IL-1)[12]. Interestingly, it has been shown that culturing of dedifferentiated chondrocytes in alginate induces re-expression of the differentiated chondrocyte phenotype[13–16]. In alginate the dedifferentiated chondrocytes recovered the capacity to produce alcian-blue-stainable pericellular matrix, but only if the chondrocytes were obtained from young animals. This capacity was not reported for immortalized cells from embryonal origin. We examined whether our immortalized cell lines derived from adult cartilage retain the capacity to produce such pericellular matrix.

To characterize the chondrocyte phenotype we assayed the production of collagens. The production of type II collagen, and especially collagen IIB, was considered as a marker for differentiated chondrocytes. Moreover, since responses to IL-1 are lost in dedifferentiated chondrocytes[15], we tested IL-1-induced suppression of proteoglycan synthesis and IL-1 induced nitric oxide (NO) synthesis.

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Our results demonstrate that it is possible to immortalize chondrocytes from mature murine cartilage, and to obtain chondrocyte cell lines with stable expression of the differentiated phenotype using this methodology. Culturing in alginate stimulated expression of differentiated phenotype by immortalized chondrocytes.

Materials and methods

CHONDROCYTE ISOLATION AND CULTURE

Small pieces were cut from articular cartilage, away from synovial tissue, of 30 male C57Bl/6 mice, which were 3 months of age. These slices were incubated overnight with 1.5 mg/ml collagenase B (Boehringer, Mannheim) at 37°C. Slices from patellar and femoral head cartilage were treated separately. The next day the isolated cells were washed and suspended into DMEM/Ham's F12 (GIBCO)+penicillin 500 U/ml (GIBCO)+streptomycin sulfate 500 μg/ml (GIBCO)+10% fetal calf serum (FCS) and seeded into 24-well culture plates.

RETROVIRAL INFECTION

The ψ2-865 cell line producing the ectopic helper-free retrovirus was provided by P. A. Sharp (Massachusetts Institute of Technology, Cambridge). This replication-defective retrovirus encodes the SV40 large T antigen and a protein conferring resistance to neomycin. Two weeks after isolation and seeding into 24-well culture plates, the primary chondrocytes clearly showed proliferation and this time-point was chosen for infection. The chondrocytes were infected for 2 h with the retrovirus supernatant, in the presence of Polybrene (Aldrich) at 8 μg/ml. After infection, fresh medium was added, and 48 h later the cultures were subjected to selection for 1 month in the presence of G418 (Geneticin; GIBCO) at 200 μg/ml. The selective medium was changed every 3–4 days. During the selection period, 16 colonies of cells showing a polygonal morphology were isolated by using cloning rings. Ten of those were derived from patellar cartilage and six from femoral head cartilage. To obtain cell lines the cells of these populations were passed at high dilution and for each of the 16 populations one resulting clone was isolated. The resulting cell lines were grown in monolayers to produce stocks that could be stored in N_2 for future experiments.

COMPARATIVE STUDIES

For validation of mRNA levels in our chondrocyte cell lines, extracts from native murine articular (patellar) cartilage were used as a standard. We studied deposition of pericellular matrix in bovine chondrocytes, derived from the methacarpophalangeal joint of cows. Slices of cartilage were cut and digested overnight in 1.5 mg/ml collagenase B (Clostridiopeptidase, Boehringer, Mannheim) at 37°C. These primary cells were either embedded in alginate beads immediately, or after 1 week culture in monolayer. Moreover, we compared our cell lines to MC615 (a kind gift from Dr F. Mallein-Gerin, Lyon), a well-characterized chondrocyte cell line that was developed following the same procedure, using murine chondrocytes from embryonal origin[5].

CHONDROCYTE CULTURE IN ALGINATE

Chondrocytes were released from monolayers by trypsinization. Isolated cells were encapsulated in alginate beads at a density of 8×10^6 cells/ml of gel. The preparation of chondrocytes in alginate beads was performed as described by Guo *et al.*[8], with slight modifications described by Häuselmann *et al.*[9]. Briefly, the cells were suspended in sterile saline containing 1.2% low-viscosity alginate gel (Keltone LV, Kelco, Chicago, IL, U.S.A.), then slowly pressed through a 22-gauge needle in a dropwise fashion into a 102 mM $CaCl_2$ solution. After instantaneous gelation the beads were allowed to polymerize further for a period of 10 min in the $CaCl_2$ solution. Thereafter they were washed with saline and finally placed in Ham's F12/ Dulbecco's modified Eagle's medium (DMEM) medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% FCS, penicillin 100 U/ml and streptomycin 100 μg/ml (Gibco, Grand Island, NY, U.S.A.).

PLASTIC EMBEDDING OF ALGINATE BEADS

For light microscopy, the beads were fixated in a 0.1 M phosphate buffered solution (pH 7.4) of 1% paraformaldehyde and 1.25% glutaraldehyde, dehydrated in alcohol, and embedded in polymethylmethacrylate (all incubations in presence of 100 mg CaCl/l). Sections (7 μm) were stained with haematoxylin and eosin and alcian blue. Histology was performed after 2 weeks of culture in alginate beads. In one experiment additional histology was taken at later time-points (1 and 2 months).

RNA EXTRACTION AND RT-PCR

After culturing immortalized chondrocytes in alginate beads for 2 weeks, the beads (2 beads per cell line) were dissolved using 55 mM sodiumcitrate pH 6.8, the cells were spun down and the RNA in the pellet was isolated using a RNeasy kit (Qiagen Inc, Valencia, CA, U.S.A.), according to the methods described by the supplier. For the study of mRNA levels in murine articular cartilage, patellae were dissected and immediately decalcified in 3.5% EDTA for 4 h at 4°C. Following decalcification, the complete articular cartilage was stripped from the underlying bone. The isolated cartilage was instantly put in TRIzol reagent (Life Technologies). The cartilage of ten patellae was pooled. The procedure of patellar cartilage isolation and RNA extraction has been described and evaluated by van Meurs *et al.*[17]. Before reverse transcription, the isolated RNA was treated with DNAse 1 (Life Technologies). The reverse transcription reaction was performed with moloney-murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies), using an oligo$(dT)_{15}$ primer (Eurogentec, Liege, Belgium). Amplification of DNA was accomplished by using Taq DNA polymerase (Life Technologies) up to a cycle number of 40. To estimate the relative mRNA levels, 5 μl samples were taken at increasing cycle numbers. The PCR products were electrophorized in 1.6% agarose gels containing ethidium bromide. The cycle number at which the product was first detected on gel was taken as a measure for the amount of specific mRNA present in the originally isolated RNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were used as an internal control. This method was validated by van Meurs *et al.*[17]. The results are expressed as the relative mRNA levels, whereby the expression in normal murine articular cartilage was arbitrarily set at 100%. For example, in normal intact murine articular cartilage the first visible PCR product of type II collagen mRNA on gel is six cycles later than the first band of GAPDH. If in one of the cell lines this difference is 10 cycles, instead of six, the relative amount of type II collagen has been $1.8^4=10.5$ times lower than in intact murine articular cartilage (theoretically in each PCR cycle the amount of cDNA is multiplied 1.8 times). Because the type II collagen level in intact murine articular cartilage is set at 100%, the relative level in that cell line is 100/10.5=9.5%. For determination of the relative mRNA levels in subconfluent monolayer and in alginate beads after 2 weeks culture, the RNA extracts of at least two different experiments were analized by RT-PCR. Aggrecan primers were used as described by Grover and Roughly[18]. The primers used to detect GAPDH and murine type I, II, and X collagen have also been described before[19]. The primers used to detect murine type II collagen discriminate between collagen IIA and collagen IIB.The upper primer is situated on exon 1 (gene position 393–413) while the lower primer is positioned on exon 4 (gene position 8815–8835). Since type IIB collagen lacks exon 2, the product size of IIB will be 177 bp while the product size of IIA (including exon 2) is 384 bp. Therefore, both IIA and IIB collagen mRNA can be detected in a single RT-PCR procedure. Primer sequences were selected using computer programs Primer (Whitehead Institute, Cambridge, MA) and oligo 4.0 (National Biosciences, Plymouth, MN) and sequence data from international data banks.

IMMUNOLOCALIZATION OF EXTRACELLULAR MATRIX COMPONENTS

To stain for type I collagen, we used a commercially available polyclonal rabbit antibody (Biogenesis, Poole, U.K.). To stain type II collagen the monoclonal antibody (IgG2a) CIICI developed by Holmdahl *et al.*[20] was used. This antibody was obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, under contract N01-HD-7-3263 from the NICHD. Type X collagen was stained with a polyclonal rabbit antibody developed by Dr A. R. Poole and Dr T. H. M. Meijers, Montreal. This antibody was raised against a human NC1 synthetic peptide unique for type X collagen. The human and mouse sequences of this peptide have only a single amino acid difference. The type X collagen polyclonal antibody specifically stains type X collagen in murine joints[19,21] and shows no cross-reactivity against collagen type I, II, IX, and XI. To stain for alkaline phosphatase cryosections were incubated with naphthol AS-BI/fast red.

After 2 weeks culture alginate beads were rapidly frozen in liquid nitrogen and stored at −70°C. Cryosections were cut on a cryostat and mounted on glass microscopic slides. Sections were dried for 30 min, fixated for 5 min in freshly prepared 4% formaldehyde and washed extensively in 0.1 M phosphate buffered saline (pH 7.4, PBS) for 15 min. All treatments of the sections had to be performed in the presence of 100 mg $CaCl_2$/liter, to prevent desintegration of the alginate matrix. To enhance the permeability of the extracellular matrix, glycosaminoglycans were removed by incubating the sections with 1% hyaluronidase (type I-s, Sigma, St Louis, MO) for 30 minutes at 37°C. Endogenous peroxidase was blocked by freshly prepared 1% (vol/vol) H_2O_2 in absolute ethanol for 30 min. Non-specific staining was blocked by incubation of the sections with 10% normal horse serum in PBS with 1% bovine serum albumin (Sigma). Sections were incubated with the corresponding biotin-labeled second antibody whereafter staining was performed with a biotin-streptavidin detection system (Vectra elite kit, Vectra, Burlington, CA, U.S.A.). Sections were counterstained with haematoxylin, dehydrated, and mounted with DPX (BDH, Poole, U.K.).

MEASUREMENT OF PG CONTENT

The amount of PGs deposited in the alginate beads by chondrocytes was determined at the end of 2 weeks culture. Two beads were used per experimental group. After dissolving the beads in sodium-citrate they were digested by papain treatment (1 mg/ml, 20 h, 60°C). One half of the digest was used for measurement of glycosaminoglycan (GAG) content using the Farndale assay[22] and the other half for measurement of DNA content. For the first purpose, 100 μl of the digest was mixed with 1 ml dimethylmethylene blue (DMB) and immediately thereafter, the glycosaminoglycan (GAG) content was measured with spectrofotometric analysis of DMB-GAG complex formation. Chondroitin sulphate from shark (Sigma, St Louis, MO) was used as a standard. Because alginate itself can react with DMB reagent, we subtracted the value of alginate beads without embedded cells.

CYTOKINE INCUBATIONS

After 2 weeks culture groups of six alginate beads were placed into 24-well culture plates. The medium supplemented with FCS was replaced by 1 ml medium plus 0.25 μg rec hIGF-1/ml (Novartis, Switzerland) to provide more defined culture conditions, as compared to FCS. The medium was replaced daily. Two days later 10 ng/ml rec mIL-1α was added and after another 2 days PG synthesis was determined. The medium supplemented with IL-1 was replaced after 1 day and the media of day 1 and 2 were used for measurement of nitric oxide (NO) production. Moreover, part of the cell lines were incubated with 15 ng TGFβ2 (Novartis, Switzerland) or 10, 50, or 100 ng BMP-2, (Genetics Institute Inc, Cambridge, MA, USA) instead of IL-1α.

MEASUREMENT OF PG SYNTHESIS

PG synthesis was measured by ^{35}S-sulphate incorporation. After culture the beads were transferred to new wells. The beads were then incubated with medium plus 10 μCi ^{35}S-sulphate/ml for 2 hours at 37°C under 5% CO_2. Thereafter the beads were rinsed extensively and the individual beads were dissolved with Lumasolve (Omnilabo, Breda, The Netherlands) during 4 h at 60°C. The ^{35}S-sulphate content of each bead was measured by liquid scintillation counting and expressed as cpm.

The loss of newly synthesized proteoglycans was studied in pulse-chase experiments. After 2 h labeling followed by extensive washing, cells in beads were cultured for another 24 h. Thereafter, the amount of incorporated label was compared with that immediately after the washing procedure.

MEASUREMENT OF NITRITE PRODUCTION

In culture media the concentration of nitrite (NO_2^-), a stable reaction product of nitric oxide (NO), was determined by the Griess reaction using $NaNO_2$ standards (Merck, Darmstadt, Germany). This was done with conditioned media collected after 1 or 2 days incubation with IL-1 (medium was changed after the first 24 h). Briefly, 100 μl of conditioned medium was mixed with 100 μl of Griess reagent (0.1% naphthylethylenediamine dihydrochloride (Sigma, St Louis, MO), 1/1 diluted with 1% sulfanilamide (Sigma) in 5% H_3PO_4) in a flat-bottomed microtiter plate (Costar, Cambridge, MA). The OD at 535 nm was measured using an ELISA plate reader.

MEASUREMENT OF DNA CONTENT

Papain-digested alginate beads were used for measurement of DNA content, to determine the amounts of cells present in beads. This was needed to enable the calculation of proteoglycan or NO production per cell. DNA was quantitated with PicoGreen dsDNA quantitation reagent (Molecular Probes, Inc., Eugene, OR) using a spectrofluorometer (FLUOstar, BMG Lab Technologies, Offenburg, Germany).

STATISTICAL ANALYSIS

Differences between experimental groups were tested using the Student's *t*-test (two-tailed).

Results

Starting from adult murine chondrocytes 16 cell lines were obtained through clonal selection. Ten lines (P1 trough P10) were derived from patellar cartilage, and six lines (H1 through H6) from femoral head cartilage. Their characteristics were determined in monolayer and in alginate beads, and compared with intact murine articular cartilage, and with a murine chondrocyte cell line from embryonal origin (MC615).

HISTOLOGY

Light-microscopical examination of sections from plastic-embedded alginate beads showed that all chondrocyte cell lines formed clusters of rounded cells (Fig. 1). No clear pericellular matrix could be demonstrated using alcian blue staining after 2 weeks or 1 month culture in alginate. However, after 2 months culture the H5 cell line had produced substantial pericellular matrix. Figure 1 (middle panel) shows the typical cell clusters and the pericellular matrix of this cell line. For comparison, the characteristic appearance of bovine primary chondrocytes after 2 months culture in alginate was also included in this figure (bottom panel). These cells had produced a clear pericellular matrix already after 2 weeks culture in alginate beads, but this feature was lost if the cells were cultured in monolayer during one week before they were placed in alginate beads (Fig. 2). This finding makes it less unexpected that the chondrocyte cell lines have difficulty in producing pericellular matrix.

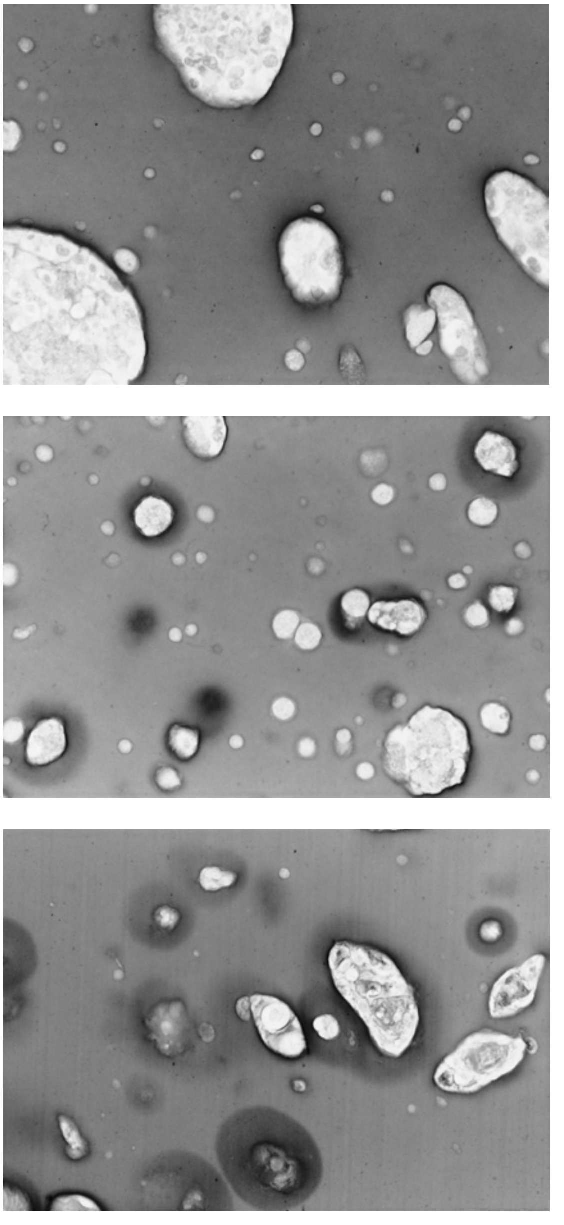

Fig. 1. Production of pericellular matrix by immortalized murine chondrocytes in alginate beads. Alcian blue-stained sections of chondrocytes cultured in alginate beads in the presence of 10% FCS. In most cell lines no clear alcian blue-stained pericellular matrix was found, neither around individual chondrocytes, nor around chondrocyte clusters. Top: cell line H1 after 2 months culture; like most of the cell lines H1 did not produce substantial pericellular matrix. Middle: cell line H5 after 2 months culture; this cell line produced a clearly visible pericellular matrix. This structure was less extensive after 1 month or 2 weeks culture. For comparison primary bovine chondrocytes were cultured under the same conditions. Bottom: bovine chondrocytes after 2 months culture in alginate. Original magnification: ×400.

MRNA LEVELS OF EXTRACELLULAR MATRIX PROTEINS

The type II collagen mRNA levels were measured after 2 weeks culture in alginate beads. Large differences were found in mRNA levels of type II collagen (Table I), which ranged between the level found in normal murine cartilage (first visible PCR product on gel six cycles later than GAPDH) and about 30 times lower levels in P1, P9, and P10. In MC615 even 1000 times lower levels were found, but still this cell line produced some type II collagen, in line with earlier studies[5]. If the cell lines were cultured in monolayer, instead of beads, the mRNA level of type II

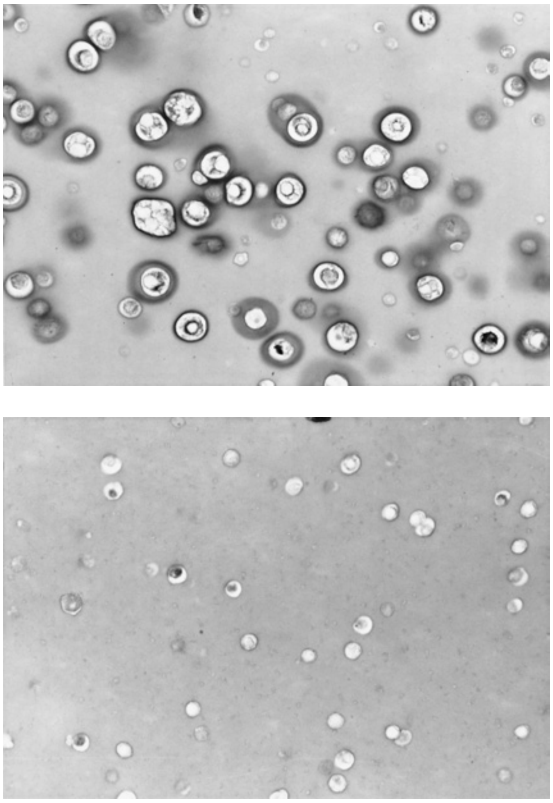

Fig. 2. Loss of chondrocyte differentiation in monolayer culture. Freshly isolated bovine articular chondrocytes were placed either directly in alginate or were cultured one week in monolayer prior to embedding them in alginate. Top: bovine chondrocytes had produced pronounced alcian blue stainable pericellular matrix after 2 weeks in alginate. Bottom: this capacity was lost after preculturing in monolayer. Original magnification: ×400.

collagen decreased in all cell lines. The results in Table I were derived from one single experiment with all cell lines included. Smaller experiments, comparing fewer cell lines, were very reproducible, differing, at highest, two cycles from the presented data.

Moreover, there were differences in the relative amounts of the IIA proform of type II collagen, characteristic of chondrocyte precursors, and the differentiated chondrocyte-specific, smaller type IIB collagen mRNA (Table I). Normal murine cartilage contains mainly the IIB type mRNA, but most cell lines contained both types, although in different proportions. The chondrocyte-specific type IIB collagen mRNA predominated in those cell lines that also showed a differentiated phenotype when tested for the other parameters. The collagen type IIA mRNA predominated in cell lines P1 and P7. If the cell lines were cultured in monolayer instead of alginate beads a shift to the immature type IIA collagen mRNA was found in most cell lines, except in the P3, P4, P9, and the MC615 cell lines.

Because an increased production of type I collagen is seen as an indicator for chondrocyte dedifferentiation, at least in human cartilage, we also analysed this mRNA (data not shown) after 2 weeks culture in alginate. Increased expression of type I collagen mRNA was never found. In most cell lines the mRNA expression of type I collagen was comparable to that in normal murine patellar cartilage (first visible band on gel two cycles later than GAPDH). In cell lines P3, P4, P10, H4, and MC615 the level was 10 times lower, and in cell lines H1, H5, and H6 this was as much as 34 times lower.

Type X collagen is highly produced in hypertrophic chondrocytes. No shift towards this phenotype was found, as all chondrocyte cell lines showed lower type X collagen mRNA expression than intact murine articular cartilage (first visible band on gel 12 cycles later than GAPDH, data not shown).

Expression of aggrecan mRNA (data not shown) was comparable to intact murine articular cartilage (first visible band on gel 12 cycles later than GAPDH) in cell lines P3, P4, P5, H1, H2, H4, and H5. Expression was three times lower in the other cell lines.

DEPOSITION OF TYPE I, II, AND X COLLAGEN IN EXTRACELLULAR MATRIX

After 2 weeks culture in alginate, cryosections of the beads were analysed for collagen production using immunolocalization.Type II collagen protein expression (Table II and Fig. 3) was found in cell lines P2, P3, P5, P6, P7, H1, H3, H4, H5, H6 and MC615, with highest amounts present in H1 and H5. Type II collagen protein levels showed no clear correlation with type II collagen mRNA levels or type IIA/IIB ratio. No type II collagen protein was found in those cell lines with the lowest mRNA levels, except the MC615 which produced some protein in spite of very low mRNA levels.

Immunolocalization also demonstrated that the collagen types I and X were produced by all cell lines. There were no clear differences in the amounts produced (data not shown). Figure 4 shows some examples. Because type X collagen staining indicates chondrocyte hypertrophy, at least in human cartilage, we stained also for alkaline phosphatase. P7 was the only cell line which showed alkaline phosphatase activity.

PG RETENTION IN ALGINATE MATRIX

Because immortalized chondrocytes appeared to produce very little alcian-blue-stainable pericellular matrix we studied the fate of newly produced PGs (data not shown). Pulse-chase experiments with ^{35}S-sulfate demonstrated that a relatively large part of the newly synthesized proteoglycans was lost from the beads. During 24 h chase after 2 h labeling and extensive washing, in all murine cell lines about 60% of incorporated label was lost. When bovine chondrocytes cultured for two weeks in alginate were tested in the same pulse-chase experiment, only 10–20% of newly synthesized proteoglycans were lost from the beads (PG synthesis per cell appeared to be 10 times higher in bovine chondrocytes, compared to murine chondrocyte cell lines). Normal loss of newly synthesized proteoglycans in murine articular cartilage *in vivo* is about 20% in a 24 h period[23]. In spite of high PG turn-over by the chondrocyte cell lines, after two weeks in culture they all had deposited about 5 μg GAG per alginate bead (16 μg when using bovine chondrocytes).

EFFECT OF IL-1 ON PG SYNTHESIS AND NO PRODUCTION

Incubation of immortalized cells in alginate beads (2 weeks pre-culture) with 10 ng IL-1α during 48 h induced a wide range of effects on proteoglycan synthesis and NO

Table I
Type II collagen mRNA levels

Cell line	Monolayer			Alginate beads		
	mRNA(%)*	Type IIA	Type IIB mRNA(%)	Type IIA	Type IIB	
P1	1	++++		3	+++	+
P2	3	+++	+	100	+	+++
P3	1	+	+++	10	+	+++
P4	3	++	++	10	++	++
P5	1	++	++	100	+	+++
P6	3	+++	+	100	++	++
P7	1	++++		31	++	++
P8	0.3	+++	+	10	+	+++
P9	0.1	++	++	3	++	++
P10	1	+++	+	3	++	++
H1	100	+	+++	324		++++
H2	10	+++	+	31	+	+++
H3	0.3	++	++	324	+	+++
H4	10	++	++	31	+	+++
H5	10	++	++	31	+	+++
H6	1	+++	+	10		++++
MC615	0.03	+++	+	0.1	+++	+

*The relative level of type II collagen mRNA was expressed as % of the basal level in intact murine articular cartilage (For details about calculations see Materials and methods section). ++++=this mRNA type is present in at least 16 times higher amounts, +++=this mRNA type is present in at least four times higher amounts, ++=equal amounts of types IIA and IIB, +=this mRNA type is present in at least four times lower amounts.

Table II
Type II collagen protein expression as revealed by immunohistochemistry

P1	–	P10	–
P2	++	H1	+++
P3	+	H2	–
P4	±	H3	++
P5	++	H4	++
P6	++	H5	+++
P7	+	H6	++
P8	±	MC615	+
P9	–		

Intensity and extensiveness of immunostaining was scored semi-quantitatively. ±=<10% of the cells is stained faintly, +=>80% of the cells is stained, but <50% is stained intensely, ++=>50% of the cells is stained intensely, but there is not much deposition outside the cells, +++=>80% of the cells is stained intensely, combined with extensive extracellular staining.

production (Table III). The effects of IL-1 on proteoglycan synthesis as measured by ^{35}S-sulfate incorporation presented in the table are from one large experiment with all cell lines included. Other experiments with less cell lines showed good reproducibility of these effects. Basal levels of proteoglycan synthesis were very similar for the different cell lines, in line with the small differences in aggrecan mRNA levels. Effects of IL-1 on proteoglycan synthesis varied between 72% suppression and 47% stimulation in the cell lines. For comparison, proteoglycan synthesis of intact murine cartilage was suppressed to about 50% of basal level after 48 h culture with the same amount of IL-1α [24].

NO production induced by IL-1 ranged from 12 to 39 nmol per 10^6 cells per 24 h. We found no clear correlation between effects of IL-1 on PG synthesis and on NO production. In Table III only the concentrations after the first 24 h are presented; the NO production in the next 24 h was comparable. If the cells were cultured in 10% FCS instead of 0.25 μg IGF-1/ml, NO production was comparable, but IL-1-induced suppression of PG synthesis in most cell lines was more pronounced, especially in the low responders.

In addition to responses to IL-1, also effects of the growth factors TGF-β1 and BMP-2 were tested after 2 weeks culture in alginate beads (data not shown). Two days exposure to 15 ng/ml TGF-β1 stimulated PG synthesis to at least two times basal values and in some cell lines to even above three times basal values. 20 ng/ml BMP-2 had no effect on PG synthesis, while 50–100 ng induced only 30–40% extra synthesis in most cell lines. For instance, the IL-1 responder H1 reacted to 15 ng TGF-β1 or 100 ng BMP-2 with 248% and 37% extra PG synthesis, respectively. However, TGF-β also strongly stimulated PG synthesis in low IL-1 responders such as P1, P3, and P4 (53%, 284% and 157% extra, respectively).

In the H1 cell line we determined for what period the cells could be cultured in monolayer without losing their characteristic pattern of responses to cytokines and growth factors (data not shown). When H1 cells were placed in alginate beads after 5 months (more than 150 cell divisions) in monolayer culture, their responses to anabolic factors like IGF-1, TGF-β, and BMP-2 and to the catabolic factor IL-1 were unchanged. Somewhere between 5 and 7 months (two passages each week) culture in monolayer all these responses decreased strongly.

Discussion

Taking into account the scores for all parameters measured, seven cell lines (P2, P5, H1, H3, H4, H5, H6) showed a phenotype comparable to differentiated chondrocytes. Of those seven, H1 and H5 showed the highest type II collagen deposition. Cell lines P1, P4 ,P7, P9, P10, H2 and MC615 can be classified as having a dedifferentiated phenotype, although P7 also had some characteristics of hypertrophic cells. Miscellaneous patterns were found in P3, P6 and P8. The large variability in expression of phenotypic markers between the cell lines could have several reasons. A possible explanation is the existence of

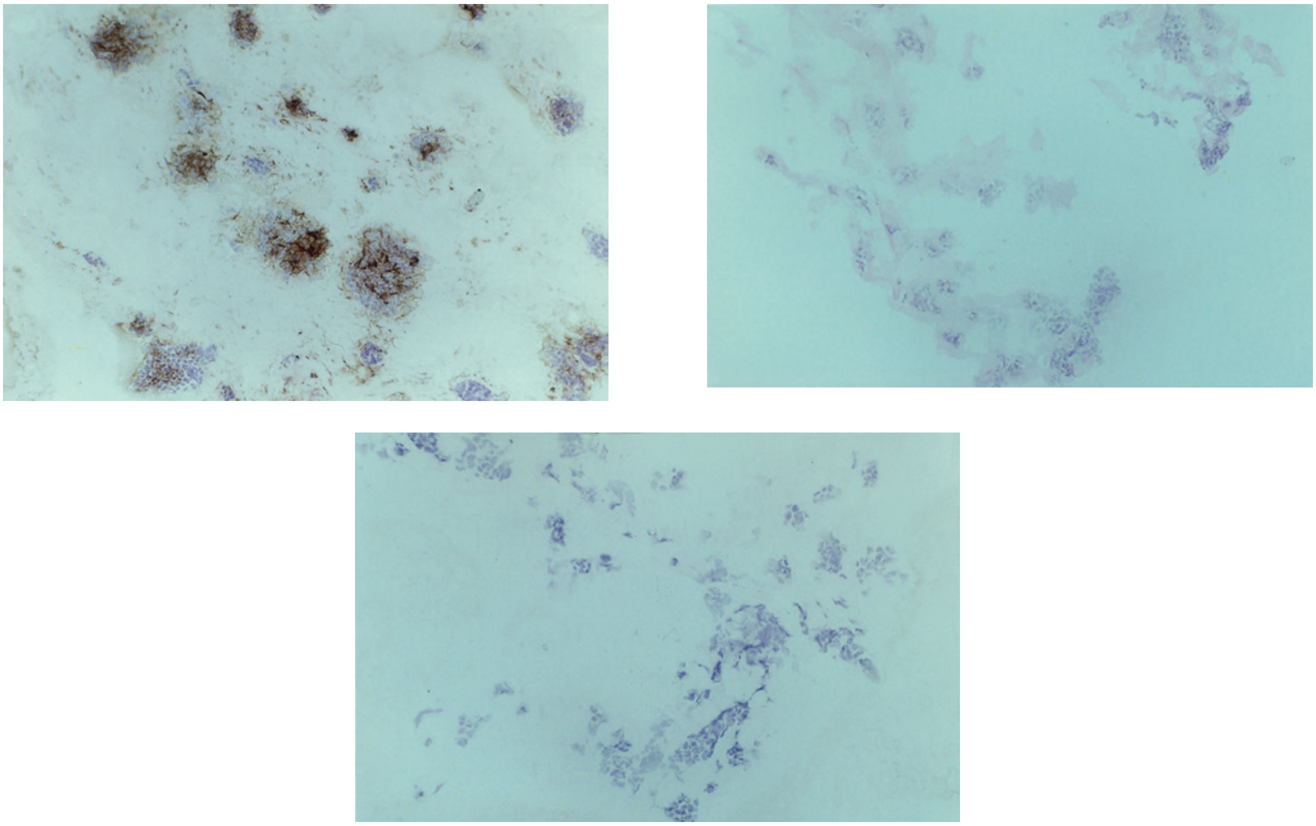

Fig. 3. Deposition of type II collagen in alginate beads. Cryosections of alginate beads containing immortalized cells after 2 weeks culture were used for immunostaining of type II collagen. Top, left: cell line H1 stained with the CIICI monoclonal antibody. Type II collagen immunostaining was found both intra- and extracellularly. Top, right: H1 stained for the isotype control for the CIICI monoclonal antibody. Bottom: line P1 stained with CIICI. Cells were counterstained briefly with hematoxylin. Original magnification: ×200.

different chondrocyte subpopulations in murine articular cartilage, which give rise to different chondrocyte cell lines. Moreover, during the time of pre-culture in monolayer of isolated primary chondrocytes before transfection, some degree of chondrocyte dedifferentiation could occur, and this degree could be different for each cell line. Immortalization appeared to stabilize the phenotype. Also, the transfection itself could be crucial for the resulting phenotype, because the number of oncogenes brought into the cell, and the place where they are incorporated into the genome, can differ.

Alcian blue staining of histological sections from alginate beads revealed that the immortalized murine chondrocytes produced little pericellular matrix. This was different from the behavior of freshly isolated bovine chondrocytes in alginate and indicated that the preservation of a differentiated chondrocyte phenotype was not totally reached. Histology corresponded with the studies of PG synthesis and loss of newly synthesized PG from the beads, which showed higher PG synthesis and longer retention of newly formed PG in the beads with bovine primary chondrocytes, compared to the murine cell lines. Immortalization and presumed concomitant changes in proliferative activity could be related to decreased matrix production and retention. Defective pericellular matrix production was reported in earlier studies using SV40-immortalized chondrocytes[4,7,25,26]. The use of mature chondrocytes instead of embryonic cells only partly solves this problem, because a clear pericellular matrix was only found in cell line H5, after 2 months culture in alginate. In order to obtain a sufficient amount of proliferating chondrocytes for retroviral infection the isolated adult chondrocytes had to be cultured on plastic for two weeks. This procedure could induce loss of the ability to produce pericellular matrix after embedding in alginate, as we showed with bovine chondrocyte cultures (Fig. 2). Which changes are responsible for this matrix deposition defect is not yet known. For instance, immortalization leads to changes in integrin expression and this could change interactions of chondrocytes with types II and VI collagen. On the other hand, certain factors in the *in vivo* cartilage matrix environment can encourage immortalized chondrocytes to produce more cartilage matrix *in vivo* than *in vitro*[25]. In spite of the lack of alcian blue-stainable pericellular matrix our immortalized murine cells had normal levels of aggrecan mRNA and deposited considerable amounts of GAG in the alginate bead, although these GAGs diffusely spread over the alginate bead. The larger cell clusters appeared to deposite alcian blue-stainable extracellular matrix within the margins of the cluster. This extracellular matrix strongly resembled the material that we found in clusters of bovine chondrocytes.

Type II collagen is a specific chondrocyte product, implicating that loss of its synthesis can be seen as a sign of chondrocyte dedifferentiation or hypertrophy. Clear type II collagen production was found in cell lines P2, P3, P5, P6, P7, H1, H3, H4, H5, and H6, but this was not always accompanied by high type collagen II mRNA levels and high type IIB/IIA mRNA ratios. If cultures in monolayer and in alginate beads were compared using collagen type II mRNA levels as a measure of differentiation, all cell lines

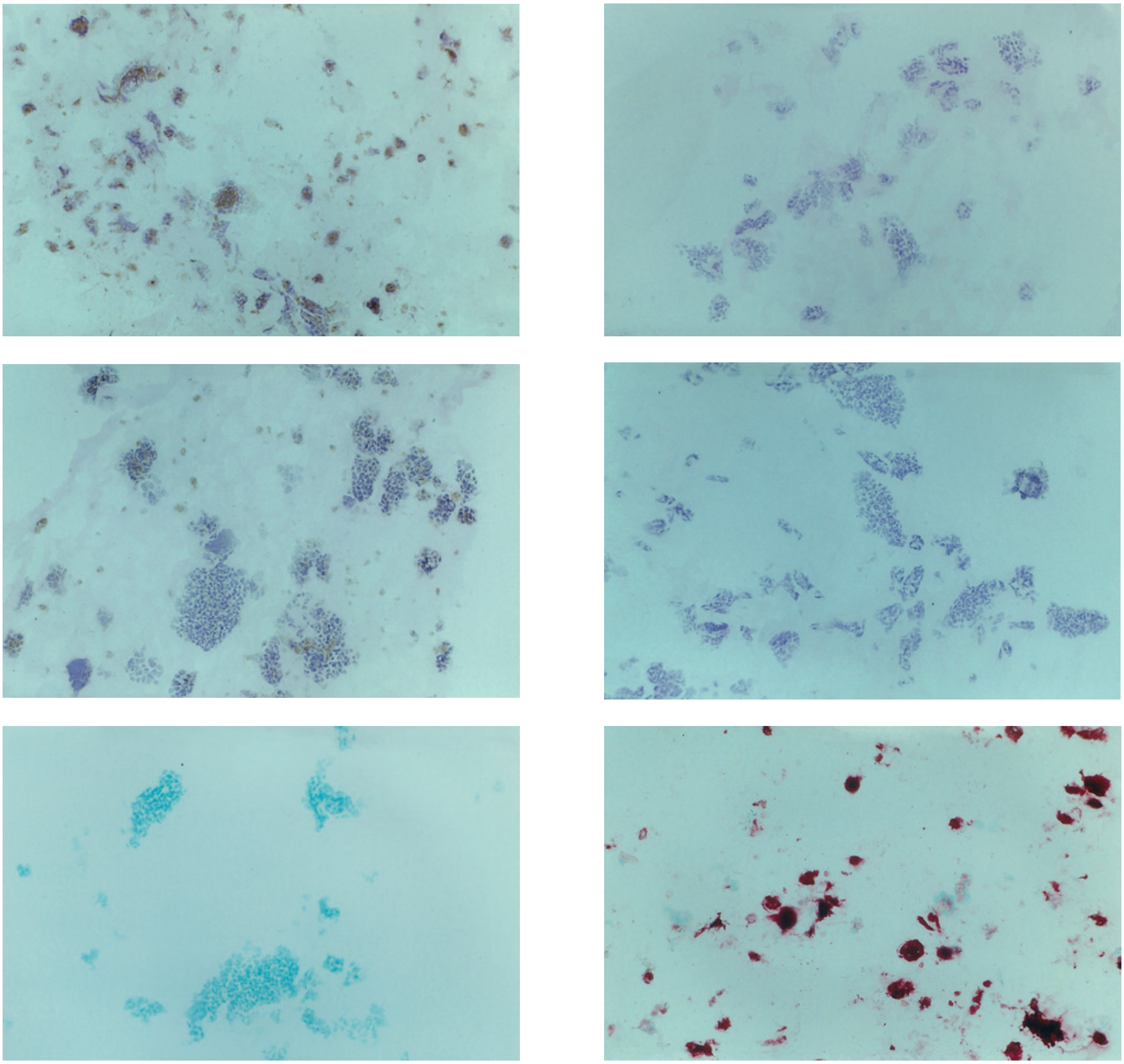

Fig. 4. Deposition of collagen types I and X, and alkaline phosphatase by immortalized chondrocytes cultured in alginate. After 2 weeks in culture cryosections were produced for immunohistochemistry. Top left: cell line H1 stained with a polyclonal anti-collagen type I antibody. Top right: negative control (without anti-collagen type I antibody). Middle left: H1 stained with a polyclonal antibody against type X collagen. Middle right: negative control (without anti-collagen type X). Bottom left: H1 stained for alkaline phosphatase. Bottom right: P7 stained for alkaline phosphatase. Cells were counterstained briefly with haematoxylin (top and middle) or methyl green (bottom). Original magnification: ×200.

except H2 showed higher mRNA levels in alginate culture. Moreover, in alginate beads most cell lines (including H2) showed a higher ratio of type IIB/Type IIA collagen mRNA, compared with monolayer cultures. This ratio was unchanged in P3, P4, P9, and MC615. Using these parameters we can conclude that culturing in alginate appeared to promote differentiation in chondrocyte cell lines. Earlier studies, using different parameters, have also shown that embedding in alginate beads preserves or promotes the diffentiated phenotype of chondrocytes[14–16].

In human articular cartilage type I and X collagen are considered as indicators of either chondrocyte dedifferentiation or hypertrophy. For characterization of the differentiation stage of our murine chondrocyte lines expression of these collagen types probably is a less specific parameter, because both collagen types have been shown to be present in normal murine articular cartilage[19]. In all lines mRNA levels of type I collagen were the same or lower, but never higher, compared to normal murine cartilage. Moreover, immunolocalization showed that type I collagen was produced by all cell lines and that there were no large differences between the amounts produced, in spite of large differences found in the other parameters. The same holds for type X collagen production. In human articular cartilage type X collagen production characterizes chondrocyte hypertrophy. Besides type X collagen

Table III
IL-1 effects on PG synthesis and NO production

Cell line	^{35}S-sulfate incorporation (cpm/10^6 cells)			NO (nmol/10^6 cells/24 h)[a]	
	Control	IL-1	% inhibition	Control	IL-1
P1	2085±318	2377±297	−14	2.0±0.6	15.8±6.2*
P2	1899±112	1500±136	21***	6.4±1.1	27.3±13.3
P3	1734±201	1491±113	14*	2.1±1.8	11.9±2.5**
P4	1477±245	1359±143	8	2.6±0.4	18.8±5.6**
P5	1258±134	855± 59	32***	8.3±3.1	15.4±4.9
P6	1498±108	1288±114	14**	1.9±1.4	17.3±2.5***
P7	1300±106	1916±134	−47***	1.1±0.6	14.9±8.6
P8	1546±102	1113±166	28***	8.5±5.3	16.3±6.3
P9	1656±174	1457±132	12*	3.0±1.8	13.4±4.6*
P10	1866±122	1568±107	16**	7.0±1.1	25.4±9.1*
H1	2003±212	560± 47	72***	1.2±0.5	19.6±8.3*
H2	1905±102	1008±107	47***	2.4±0.2	14.8±4.7*
H3	1896±102	1081±105	43***	4.7±1.7	24.8±15.3
H4	1788±177	892±121	50***	5.8±5.2	21.1±9.3
H5	1867±213	1104±135	41***	12.9±4.3	39.1±16.9
H6	2121± 54	1595±125	25***	13.3±4.1	30.1±9.4*
MC615	2100±166	1938±114	8	1.2±0.4	1.3±0.6

After 2 weeks pre-culture the immortalized chondrocytes in alginate beads (six beads per experimental group) were exposed to 10 ng/ml recombinant murine IL-1, during 48 h. Effects of IL-1 on proteoglycan synthesis are from one representative experiment (n=6); Effects on nitric oxide production are derived from three different experiments (n=3). Results are presented as means with standard deviations.

[a]NO production was determined by measuring the concentrations of the stable end product nitrite in conditioned media after 24 h incubation with or without IL-1.

Statistical significance of responses to IL-1: *P<0.05, **P<0.01, ***P<0.001.

production, another feature of hypertrophic chondrocytes is production of alkaline phosphatase, and this was found only in cell line P7. This indicated that the finding of type X collagen production in all cell lines does not necessarily mean that they have originated from hypertrophic chondrocytes or that they have gone into terminal differentiation caused by the culturing or immortalization procedures.

Only a few cell lines consistently showed IL-1-induced suppression of PG synthesis of about 50%, comparable with suppression seen in intact murine cartilage after *ex vivo* IL-1 exposure[24]. All these cell lines had originated from femoral head cartilage (H1, H2, H3, H4, H5). In two of the cell lines derived from patellar cartilage (P1 and P7) PG synthesis was even stimulated by IL-1, indicating that they might be more fibroblast-like. In lines P3, P4, P6, P9, P10, and MC615 IL-1-induced suppression of PG synthesis was less than 20%; possibly some of these cell lines could serve as models for defective IL-1 signaling. Low responses to IL-1 could not be attributed to low basal cell activity (PG synthesis was normal) or to cell anergy (NO production was stimulated and reactions to growth factors were normal). Strong IL-1-induced suppression of PG synthesis did not always predict a differentiated chondrocyte phenotype. On the other hand, high type II collagen production and predomination of IIB mRNA type was always accompanied by high responsiveness to IL-1 with regard to suppression of PG synthesis. The suppressive effect of IL-1 on matrix production by immortalized chondrocytes appeared to be a very stable characteristic, and if this was lost after more than 5 months culturing in monolayers, the responses to anabolic factors[27–29] also waned.

In conclusion, a set of characterized murine articular chondrocyte cell lines has been developed. Chondrocytes of the cell lines with the most differentiated phenotype can be embedded in alginate beads to be used as a model for cartilage studies. The other cell lines could be useful in studies of mechanisms involved in chondrocyte differentiation.

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